by means of Cellulofine GCL-300 sf gel filtration column chromatography. Subsequently, each fraction containing enzyme activity was subjected to SDS-PAGE. This analysis showed the presence of an approximately 60 kDa protein exhibiting enzyme activity. This molecular mass was equal to that of Nacrein.

To determine the amino terminal sequence, PVDF membrane transferred a 60 kDa protein was subjected to a sequence analyzer. The sequence of the first 11 amino acids agreed with that of Nacrein. Based on the results described above, we have concluded that the carbonic anhydrase of the prismatic layer is Nacrein. This result is unexpected,

Recently, the cDNA of a Nacreion-like protein called N66 was cloned from *Pinctada maxima.* RT-PCR analysis of the N66 mRNA revealed that this gene is transcribed in the dorsal region of the mantle, which is responsible for nacreous layer formation, and in the mantle edge, which is responsible for prismatic layer formation (Kono et al., 2000). These results are in agreement with the conclusions of the present report.

The presence of Nacrein in both the aragonite nacreous and calcite prismatic layers is suggestive with regard to the role of the Gly-Xaa-Asn repeat. We assume that this repeat is not related to the regulation of a crystal polymorphism of calcium carbonate. Recently, a nacreous layer-specific new matrix protein family was isolated from the EDTA-insoluble matrix of the nacreous layer of *Pinctada fucata,* and it was shown that this protein family designated N16 (N16-1,2,3) induces an aragonite crystalline layer (Samata et al., 1999). Based on its amino acid sequence, which is in agreement with N16-3 except that residue 58 is N, Pearlin also belongs to this family (Miyashita et al., 2000).

It seems likely that Nacrein is involved in the regulation of crystal growth and/or morphology via an interaction between the Gly-Xaa-Asn repeat and certain crystal faces or via coordination with another matrix protein(s). Soluble protein(s) that regulate calcite crystal growth or shape by means of an interaction with a calcite crystal surface are already known in shells (Walters et al., 1997) and sponge (Aizenberg et al., 1995). Biochemical characterization of these proteins, however, has not yet been carried out.

LITERATURE CITED

Aizenberg, J., J. Hanson, M. Ilan, L. Leiserowitz, T. F. Koetzle, L. Addadi & S. Weiner. 1995. Morphogenesis of calcitic sponge spicules: a role for specialized proteins interacting with growing crystals. The Federation of American Societies for Experimental Biology Journal 9:262–268.

Belcher, A. M., X. H. Wu, R. J. Christensen, P. K. Hansma, G. D. Stucky & D. E. Morse. 1996. Control of crystal phase switching and orientation by soluble mollusc-shell proteins. Nature 381:56–58.

Benesch, R. 1984. Carbonic anhydrase and calcification. Annals New York Academy of Science 429:457–458.

Boer, H. H. & J. Witteveen. 1980. Ultrastructural localization of carbonic anhydrase in tissue involved in shell formation and ionic regulation in the pond snail *Lymnaea stagnalis.* Cell and Tissue Research 209:383–390.

Cameron, J. N. 1979. Excretion of CO_2 in water-breathing animals. Marine Biology Letters 1:3–13.

Davenport, H. W. 1945. The inhibition of carbonic anhydrase by thiophene-2-sulfonamide and sulfanilamide. Journal of Biological Chemistry 158:567.

Davis, R. P. 1959. The kinetics of the reaction of human erythrocyte carbonic anhydrase. II. The effect of sulfanilamide, sodium sulfide and various chelating agents. Journal of the American Chemical Society 81:5674–5678.

Davis, R. P. 1961. Carbonic anhydrase. Pp. 545-562 in The Enzymes. 2nd ed.

Falini, G., S. Albeck, S. Weiner & L. Addadi. 1996. Control of aragonite or calcite polymorphism by mollusk shell macromolecules. Science 271:67–69.

Forster, R. E, S. Nioka, R. P. Henry, S. J. Dogson & B. T. Storey, 1986. Lung carbonic anhydrase. Progress in Respiration Research 21:41–46.

Freeman, J. A. 1960. Influence of carbonic anhydrase inhibitors on shell growth of a fresh-water snail, Physa heterostropha. Biological Bulletin 118:412–418.

Freeman, J. A & K. M. Wilbur 1948. Carbonic anhydrase in molluscs. Biological Bulletin 94:55–59.

Hare, P. E. 1963. Amino acids in the proteins from aragonite and calcite in the shells of Mytilus californianus. Science 139:216–217.

Henry, R. P. 1984. The role of carbonic anhydrase in blood ion and acid-base regulation. American Zoologist 24:241–253.

Henry, R. P. 1996. Multiple roles of carbonic anhydrase in cellular transport and metabolism. Annual Review of Physiology 58:523–538.

Kakei, M. & H. Nakahara. 1996. Aspects of carbonic anhydrase and carbonate content during mineralization of the rat enamel. Biochemica et Biophysica Acta 1289:226–230.

Kiese, M & A. B. Hasting. 1940. Factors affecting the activity of carbonic anhydrase. Journal of Biological Chemistry 132: 281–292.

Kono, M., N. Hayashi & T. Samata. 2000. Molecular mechanism of the nacreous layer formation in *Pinctada maxima.* Biochemical and Biophysical Research Communications 269:213–218.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685.

Lowenstam, H. A. 1981. Minerals formed by organisms. Science 211:1126–1131.

Lowenstam, H. A & S. Weiner. 1989. On Biomineralization. Oxford University Press: New York. 324 pp.

Mann, S. 1988. Molecular recognition in biomineralization. Nature 332:119–124.

Medakovic, D & L. Lucu. 1994. Distribution of carbonic anhydrase in larval and adult mussels *Mytilus edulis* Linnaeus. Periodicum Biologorum 96:452–454.

Miyamoto, H., T. Miyashita, M. Okushima, S. Nakano, T. Morita & A. Matsushiro. 1996. A carbonic anhydrase from the nacreous layer in oyster pearls. Proceedings of the National Academy of Sciences USA 93:9657–9660.

Miyashita, T., Y. Takagi, H. Miyamoto, E. Nishikawa & A. Matsushiro. 2000. Complementary DNA cloning and characterization of Pearlin, a new class of matrix protein in the

nacreous layer of oyster pearls. Marine Biotechnology 2: 409–418.

Samata, T., N. Hayashi, M. Kono, K. Hasegawa, C. Horita & S. Akera. 1999. A new matrix protein family related to the nacreous layer formation of *Pinctada fucata*. FEBS Letters 462:225–229.

Tashian, R. E. 1989. The carbonic anhydrase: widening perspectives on their evolution, expression and function. BioEssays 10:186–192.

Val, A. L. 1996. Carbonic anhydrase: a multigene-multifunctional enzyme. Anais da Academia Brasileira de Ciencias 69:565–573.

Walters, D. A., L. Smith, A. M. Belcher, G. T. Paloczi, G. D. Stucky, D. E. Morse & P. K. Hansma. 1997. Modification of calcite crystal growth by abalone shell proteins: an atomic force microscope study. Biophysical Journal 72: 1425–1433.

Watabe, N. 1984. Shell. Pp. 448–485 in J. Bereiterhahan, A. G. Matoltsy & K. S. Richards (eds), Biology of the Integument. Volume 1. Invertebrates. Springer-Velag: Berlin.

Wilbur, K. M & L. Jodrey. 1955. Studies of shell formation. V. The inhibition of shell formation by carbonic anhydrase inhibitors. Biological Bulletin 108:82–112.

The Veliger 45(3):256–258 (July 2, 2002)

THE VELIGER

Thin Layer Chromatographic Analysis of Lutein and β-carotene in *Biomphalaria glabrata* Maintained on a High Fat Diet

YONGHYUN KIM[1], BERNARD FRIED[2,3] AND JOSEPH SHERMA[1]

Department of Chemistry[1] and Department of Biology[2], Lafayette College, Easton, Pennsylvania 18042, USA

Abstract. Thin layer chromatographic analysis was used to determine lutein and β-carotene in *Biomphalaria glabrata* snails maintained on a high fat diet of hen's egg yolk. The mean values of lutein in snails on a Romaine lettuce diet were approximately ×3 and ×2.5 at 14 and 20 days postculture, compared to those of snails maintained on the yolk diet. Likewise, the mean values of β-carotene for snails on the lettuce diet were approximately ×1.5 and ×6.6 at 14 and 20 days, respectively, compared to those from the snails on the yolk diet. The only significant differences in values (Student's t-test, $P < 0.05$) was at day 20 at which time the mean percent of β-carotene in the snails on the high fat diet was significantly reduced compared to snails on the lettuce diet. The concentration of lutein in the lettuce was about ×3.5 that in the egg yolk. The concentration of β-carotene in the lettuce was ×8 that in the egg yolk. In general, the concentration of these lipophilic pigments in *B. glabrata* reflected the content of lutein and β-carotene in the lettuce and egg yolk diets.

INTRODUCTION

Numerous studies have reported the use of a hen's egg yolk diet to observe nutrition in uninfected *Biomphalaria glabrata* (Say, 1816) and snails infected with larval schistosomes and echinostomes (see reviews in Fried & Sherma, 1990, 1993). These studies have observed mainly the effects of the egg yolk diet on the lipid content of the snails (Fried & Sherma, 1990, 1993), although a recent study by Kim et al. (2001) has examined the effects of this diet on the carbohydrate content of the snail. Because effects of the diet on lipophilic pigments, i.e., lutein and β-carotene, are not available, this study examined these pigments in snails maintained on hen's egg yolk.

MATERIALS AND METHODS

Twenty juvenile *Biomphalaria glabrata* snails, about 7 mm in shell diameter, were obtained from Dr. Fred Lewis, Schistosomiasis Laboratory, Biomedical Research Institute (Rockville, Maryland, USA). Groups of 10 snails were maintained at 23–24°C in aerated glass containers each containing 800 mL of artificial spring water (ASW) prepared as described by Ulmer (1970). One culture of 10 snails was fed *ad libitum* on boiled Romaine leaf lettuce (L diet). The other culture was fed the hen's egg yolk diet *ad libitum*, supplemented with 500 mg of Romaine lettuce once a week (Y-L diet), as described in Beers et al. (1995). Food and water were changed twice weekly in all cultures.

For TLC analysis, the whole-body of five individual snails (n = 5) was prepared for both Y-L and L diets at 14 and 20 days after the cultures were started. To do this, the shell of each snail was gently crushed with a hammer, and the snail body was removed with forceps. Each whole-body was homogenized in 2 mL of acetone in a glass homogenizer. The pellet was washed twice with acetone (100 μL), and the washings were combined with the supernatant. The combined supernatant was evaporated to dryness under nitrogen and then reconstituted with 200 μL or 300 μL of heptane, as necessary for the scan areas of at least one sample zone to be bracketed within the scan areas of the standard zones in the TLC analysis. Single samples (n = 1) of the hen's egg yolk (200 mg) and the Romaine lettuce (200 mg) were extracted in acetone and prepared for TLC analysis as described for the snail bodies.

The standards used for TLC analysis were lutein and β-carotene (Sigma, St. Louis, Missouri). The solid standards were weighed on an analytical balance and diluted with dichloromethane to prepare standard solutions of 0.0100 μg μL^{-1} for both lutein and β-carotene. TLC analyses were performed on Merck (EM Science, Gibbstown, New Jersey) 10 cm × 20 cm chemically bonded C-18 silica gel plates with concentrating zone (RP-18F_{254S}, Art. 15498). Plates were prewashed by development to the top with dichloromethane-methanol (1:1) and dried in air in a fumehood. The standards (4.00, 8.00, 12.0, and 16.0 μL for each standard) and 1.00–8.00 μL of the reconstituted samples were applied in separate lanes in the concentrating zone by means of a 10-μL Drummond (Broomall, Pennsylvania) digital microdispenser in a dark room with minimum lighting. The applied solutions were dried in

[3] Author to whom correspondence should be addressed: Telephone: 610-330-5463, Fax: 610-330-5705, e-mail: friedb@lafayette.edu